Supporting information for:

Differentiated roles for MreB-actin isologues and autolytic enzymes in *Bacillus subtilis* morphogenesis

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Supplementary Experimental procedures

General methods

DNA manipulations and *E. coli* DH5α transformations were carried out using standard methods (Sambrook *et al.*, 1989). Plasmid DNA and PCR fragments were purified using the Qiaprep spin miniprep kit (Qiagen) or the Qiaquick PCR purification kit (Qiagen), respectively. Transformation of competent *B. subtilis* cells was performed using an optimized two-step starvation procedure as previously described (Anagnostopoulos & Spizizen, 1961; Hamoen *et al.*, 2002). Nutrient agar (NA, Oxoid) was used for routine selection and maintenance of both *B. subtilis* and *E. coli* strains. For *B. subtilis*, cells were grown in Luria–Bertani (LB), CH or SMM defined minimal medium (Anagnostopoulos & Spizizen) containing 0.5% xylose or 1 mM IPTG when required, unless stated otherwise. For *E. coli*, cells were grown in LB medium. Supplements were added when necessary as required: 20 μg ml^{-1} tryptophan, 100 μg ml^{-1} ampicillin, 5 μg ml^{-1} chloramphenicol, 5 μg ml^{-1} kanamycin, 50 μg ml^{-1} spectinomycin, 0.75 μg ml^{-1} erythromycin and 10 μg ml^{-1} tetracycline.

Strain construction

Deletion strains

Deletion of the *ftsE, ftsX* and *ftsEX* (*B. subtilis* strains 4503, 4501 and 4502, respectively) was accomplished by double crossover of a kanamycin marker. Regions of DNA upstream and downstream of *ftsE, ftsX or ftsEX* (~3 kb each side) were PCR-amplified, using primers RevA1/RevA2 and ForA, and ForB1/ForB2 and RevB, respectively (Table S4). The kanamycin/neomycin marker was cut out of plasmid pBEST501. The PCR fragments and the kanamycin/neomycin marker were digested, ligated, and the ligation product was directly transformed to competent Bs168CA cells. All chromosomal integrations were verified by PCR, restriction digestion, and sequencing.

Fluorescent fusions

CwlO-GFP$_{sf}$

GFP$_{sf}$ was amplified by PCR from the pUC57-GFP$_{sf}$ plasmid DNA using primers GFP-sf-FEcoRI and GFPsf-RevSpeI-NotI, and then cloned between the EcoRI and NotI sites of plasmid pSG1728-CwlO, creating pSG-cwlO-gfp$_{sf}$. The resulting plasmid was used to transform Bs168CA *ΔwprA Δepr* (PDC538), with selection for spectinomycin resistance, to generate the strain PDC528, in which the *gfp$_{sf}$*-fused to *cwlO* is expressed from the xylose inducible promoter *P$_{xyl}$* at the *amyE* locus on the *B. subtilis* chromosome. Disruption of *amyE* was confirmed using a starch plate assay (Cutting, 1990), and the correct integration of the inserts at the *amyE* locus was confirmed by PCR. The GFP variant used was superfolder GFP (Pedelacq *et al.*, 2006), which is being shown to be functional in the periplasm following Sec export (Dinh & Bernhardt, 2011).

FtsEX-GFP$_{sf}$

GFPsf was amplified by PCR from the pUC57-GFPsf plasmid DNA using primers GFP-sf-FEcoRI and GFPsf-RevSpeI-NotI, and then cloned between the EcoRI and NotI sites of plasmid pSG1728-ftsEX, creating pSG-ftsEX-gfp$_{sf}$. The resulting plasmid was used to

transform Bs168CA, with selection for spectinomycin resistance, to generate the strain PDC534, in which the *gfp$_{sf}$*-fused to *ftsX* is expressed from the xylose inducible promoter *P$_{xyl}$* at the *amyE* locus on the *B*. s*ubtilis* chromosome. Disruption of *amyE* was confirmed using a starch plate assay, and the correct integration of the inserts at the *amyE* locus was confirmed by PCR.

Epitope tagging

CwlO-Flag

To construct pMUTin-'cwlO-flag a fragment containing the last 550 bp of the *cwlO* orf was amplified by PCR with the primers cwlOCterFHindIIIFlag and cwlOrevKpnIFlag from the wild-type strain 168 genomic DNA, digested with HindIII and KpnI, and inserted into the corresponding HindIII-KpnI sites of pMUTin-flag plasmid. The resulting plasmid was used to transform PDC538, with selection for erythromycin resistance, to generate the strain PDC609, in which the flag-fused to *cwlO* is expressed from the native promoter at the *native cwlO* locus on the *B*. s*ubtilis* chromosome. The newly generated strain showed a wt phenotype, indicating that the flag fusion is fully functional. The correct integration of the plasmid at the native locus was confirmed by PCR.

Inducible expression strains

amyE::P$_{xyl}$-cwlO

cwlO was amplified by PCR from the wild-type strain 168 genomic DNA using primers CwlO-FXhoI and cwlORev-EcoRI, then cloned between the *Xho*I and *EcoRI* sites of plasmid pSG1728, creating pSG1728-cwlO. The resulting plasmid was used to transform Bs168CA, with selection for spectinomycin resistance, to generate the strain PDC567, in which *cwlO* is expressed from the xylose inducible promoter *P$_{xyl}$* at the *amyE* locus on the *B*. s*ubtilis* chromosome.

aprE::P$_{spac}$-lytE

lytE was amplified by PCR from the wild-type strain 168 genomic DNA using primers LytEFXmaI and LytERevEcoRI, then cloned between the *XmaI* and *EcoRI* sites of plasmid pAPNC213-erm, creating pAPNerm-P$_{spac}$-lytE. The resulting plasmid was used to transform Bs168CA, with selection for erythromycin resistance, to generate the strain PDC620, in which *lytE* is expressed from the IPTG inducible promoter *P$_{spac}$* at the *aprE* locus on the *B*. s*ubtilis* chromosome. The correct integration of the inserts at the *aprE* locus was confirmed by PCR.

aprE::P$_{xyl}$-cwlO, aprE::P$_{xyl}$-ftsEX and aprE::P$_{xyl}$-lytE

cwlO, *ftsEX* and *lytE* orfs were amplified by PCR from the corresponding plasmids pSG-P$_{xyl}$-cwlO, pSG-P$_{xyl}$-ftsEX-gfp$_{sf}$ and pSG-P$_{xyl}$-P$_{wt}$-LytEmcherry, respectively, using primers P$_{xyl}$-FsphI and amyEtoAprERevBamHI (or LytE-revSacI, in the case of LytE), then cloned between the SphI and BamHI (or SacI, in the case of LytE) sites of plasmid pAPNC213-ery, creating the plasmids pAPNC-P$_{xyl}$-cwlO, pAPNC-P$_{xyl}$-ftsEX and pAPNC-P$_{xyl}$-Pwt-LytE. The resulting plasmids were used to transform the corresponding deletion mutant strains, with

selection for erythromycin resistance, to generate strains PDC639, PDC635 and PDC702 respectively, in which the different *orfs* are expressed from the xylose inducible promoter P_{xyl} at the *aprE* locus on the *B*. s*ubtilis* chromosome. The correct integration of the inserts at the *aprE* locus was confirmed by PCR.

B-only, BL-only and BH-only strains (PDC660, YK1119 and PDC643)

BL-only strain (YK1119) was already constructed as described in Kawai *et al.,* 2011. *B. subtilis* strain YK1012 (*Δmbl ΔmreBH*) was transformed with chromosomal DNAs corresponding to *amyE* insertions of overexpression constructs for *mreB* and *mreBH*, with selection for chloramphenicol resistance (*amyE::*P_{spacHY}*-mreB* and *amyE::*P_{spacHY}*-mreBH*), respectively (Kawai *et al.*, 2011). The resulting strains were subsequently transformed with chromosomal DNA from strain YK1119, with selection for kanamycin resistance on NA plates supplemented with 20 mM Mg^{+2}.

Resulting strains PDC660, YK1119 and PDC643 were then transformed with chromosomal DNA from strains PDC639, PDC635 and PDC702 , with selection for erythromycin and spectinomycin, in the presence of xylose on NA plates supplemented with 20 mM Mg^{+2} and/or IPTG as required, to generate strains PDC642, PDC650, PDC651, PDC662, PDC664, PDC659, PDC678, PDC688 and PDC697.

Two-hybrid plasmids

ftsE, *ftsX* and *ftsEX* orfs were amplified by PCR from the wild-type strain 168 genomic DNA using primers ForEXbaI, RevEKpnI, ForXXbaI and RevXKpnI, and then cloned between the XbaI and KpnI sites of plasmids pUT18 and pKT25, creating the plasmids pUT18-ftsE, pUT18-FtsX, pUT18-FtsEX, pKT25-ftsE, pKT25-ftsX and pKT25-ftsEX. Corresponding plasmids pairs were used to co-transform the *E. coli* BTH101 strain for 2-hybrid analysis.

Microscopic imaging

For fluorescence microscopy, cells were grown to mid-exponential phase at 30°C or 37°C and mounted on microscope slides covered with a thin film of 1.2% agarose. See figure legends for specific growth conditions employed for each experiment. Fluorescence microscopy was carried out using Zeiss Axiovert 200M, Nikon Eclipse Ti-U, spinning disk confocal microscope. The images were acquired with Metamorph 6 (Molecular Devices, Inc) and FRAP-AI 7 (MAG Biosystems) software, and analyzed using ImageJ v.1.44o (National Institutes of Health). Images from a single focal plane were deconvolved using the 'No Neighbours' algorithm from the Metamorph software package. When required, cells were incubated in the presence of the membrane dye FM5-95 (90 μg ml^{-1}, Molecular Probes) prior to microscopic examination.

Sample preparation for microscopy

For sample preparation, overnight pre-cultures of *B. subtilis* were grown in CH medium supplemented with 20 mM $MgSO_4$ (CH-Mg) and appropriate antibiotic selection, from freshly isolated colonies on plates. Day cultures were performed by diluting pre-culture to an OD600 of 0.02 in CH-Mg and grown at 30°C. Expression of fluorescent CwlO-GFP fusion was induced by addition of xylose to 0.3%. Samples for microscopic observation were taken at mid-exponential phase and immobilized on 1.2% agarose-coated microscope slides.

Protoplast preparation for microscopy

Cells of strains PDC528 (wt, CwlO-GFP_{sf}) and PDC560 (*ΔftsX::neo*, CwlO-GFP_{sf}) were grown in CH media in the presence of 0.5% xylose. Cells were harvested and re-suspended in CH-MSM media in the presence of 0.5% xylose. Cells were protoplasted by incubation with 0.5 mg ml^{-1} lysozyme during 30 min at 30°C. After CW removal, the protoplasts suspensions were split in two. One half was treated with proteinase K (10 µg ml^{-1}) for 30 min.

Cell measurements

Cells from strains included in table I, constitutively expressing soluble/cytosolic GFP protein (*aprE::*P_{rpsD}*-gfp*) were grown in LB media at 37°C and samples were taken at different time points along the growth curve. Cells were imaged by epifluorescence microscopy using an Axiovert M200 microscope (Zeiss, Oberkochen, Germany) with a 300 W lambda light source (Sutter Instrument Company, California, USA) and a Zeiss x 100 plan-neofluar oil immersion objective lens (1.3 numerical aperture). Images were captured on a 1395 x 1040 pixel CoolSNAP HQ camera (Photometrics, Ottobrunn, Germany) controlled by Metamorph software version 6.1r3 (Universal Imaging Corporation, Marlow, UK). Image analysis was performed using the open source Cell Profiler software and consisted of the following two successive steps: (i) identification of cell contour by a segmentation pipeline of fluorescence images; (ii) automatic measurement of several cell characteristics: cell length and width, perimeter and area of cells. For each image, ~100-300 cells were identified and analyzed. For each strain and time point >1000 cells were analyzed.

Cell fractionation and immunoblotting

The generous gift from K. Devine's laboratory of a polyclonal antibody raised against the native CwlO protein allowed us to detect it in cell fractionation experiments. In order to be able to perform pull-down experiments we constructed an epitope-tagged version of CwlO fused to the Flag tag. The CwlO-Flag fusion was expressed from the native chromosomal *cwlO* locus (strain PDC609). Flag epitope was fused to the carboxyl-terminal part of CwlO. To increase the stability of the CwlO-Flag bait, all pull down experiments were performed in a *wprA epr* double mutant background. The growth rates and cell shapes of these strains were indistinguishable from that of the wild type indicating that the fusion protein is functional.

When cells reached mid-exponential phase, cultures (50 ml) were collected by centrifugation (8,000 × g for 10 min at 25°C). Culture supernatants' protein content (S) was recovered by cold-acetone precipitation. Five volumes of cold acetone were added to 5 ml of culture

supernatant and incubated at -20°C for 1 h. Then, samples were collected by centrifugation (10,000 x g for 20 min at 4°C). Pellets were washed with 70 % cold-ethanol and air dried, before re-suspending the protein pellet in 0.5 ml of Tris buffer (100 mM Tris-HCl pH 7.5, 1x complete protease inhibitor).

Culture pellets were re-suspended in 4 mL 1× SMM buffer [0.5 M sucrose, 20 mM $MgCl_2$, 20 mM maleic acid), pH 7]; 250 µL 10 mg ml^{-1} lysozyme (Sigma), and 50 µL complete protease inhibitor (EDTA-free, Roche) were added to cell suspensions and incubated at 37°C for 1 h with gentle shaking. Then cultures were split into two (2x 2 ml). First half constituted the total fraction (T). Protoplasts from the second half were collected by centrifugation. Supernatants (2 ml) were collected to constitute the CW fraction (CW). Cell membranes and cytoplasmic fractions were obtained from the protoplasts' pellets. Pellets were re-suspended in 2 ml of Tris-buffer (100 mM Tris-HCl, pH 7.5, 1x complete protease inhibitor) and sonicated until a clear solution was obtained. Membrane fraction pellets (M) were collected by centrifugation (50,000 x g for 40 min at 4°C) and supernatants were also kept as cytoplasmic fractions (C). Membrane pellets were re-suspended in 2 ml of the Tris buffer. 10 µg of total protein from each extract was separated on a 4-12% SDS-PAGE gradient gel (Novex, Life technologies). Proteins were transferred to a PVDF membrane (Amersham Hybond-P) and the membrane was blocked with 5% milk in PBST (PBS, 0.1% Tween-20) for 3 h. The membrane was incubated with appropriate antibodies (anti-Flag or anti-CwlO antibodies (1:10,000 or 1:3000 respectively in PBST)) o-n at 4°C temperature. The membrane was washed three times with PBST for 10 minutes. Following the wash, the membrane was incubated with rabbit anti-mouse or goat anti-rabbit antibodies conjugated with HRP (Sigma, A9044) (1:10,000 in 5% milk in PBST) for 1 hour at room temperature. Finally, the membrane was washed three times as above and developed using the Pierce ECL 2 Western Blotting substrate reagent. Chemiluminescence was detected using an ImageQuant LAS4000mini GE Healthcare system.

Formaldehyde Cross-Linking and Pull Down of CwlO Complexes

Cross-Linking and Pull Down experiments were performed with some modifications as described by Sham *et al* 2011. Briefly, cultures (400 mL) of strains PDC612 (Bs168CA *ΔwprA::hyg Δepr::tet ΩcwlO-FLAG amyE::*P_{xyl}*-ftsEX-gfp*) and PDC613 (Bs168CA *ΔwprA::hyg Δepr::tet amyE::*P_{xyl}*-ftsEX-gfp* parent negative control) were grown exponentially to OD600 ~ 0.5. Cells were collected by centrifugation (8,000 × g for 10 min at 25°C). Cell pellets were washed with 18 mL 1× PBS at 25°C, and cells were collected again by centrifugation (8,000 × g for 5 min at 4°C). Residual supernatants were removed. Washed pellets were suspended in 19 mL 1× PBS, to which 1200 µL 37% of formaldehyde solution (Sigma) were added. Mixtures were incubated at 37 °C for 1 h. Cross-linking reactions were quenched by the addition of 4 mL 1.0 M glycine followed by incubation for 10 min at 25°C. Cells were collected by centrifugation (8,000 × g for 10 min at 4°C), washed with 20 mL 1× PBS at 25°C, and centrifuged again. Residual supernatants were removed using a fine pipette tip. Pellets of cross-linked cells were re-suspended in 5 mL 1× SMM buffer [0.5 M sucrose, 20 mM $MgCl_2$, 20 mM maleic acid), pH 7]; 250 µL 10 mg ml^{-1} lysozyme (Sigma), and 50 µL complete protease inhibitor were added to cell suspensions and incubated at 37°C for 1 h with gentle shaking. Protoplast formation was monitored by phase-contrast microscopy. Protoplasts were collected by centrifugation (8,000 × g for 10 min at 4°C). Pellets were then suspended in 5 mL buffer H (20 mM Hepes, pH 8, 200 mM NaCl, 1 mM DTT, 1 x complete protease inhibitor) at 4°C. After mixing, 5 µL 0.1 M $MgCl_2$, 5 µL 0.1 M

CaCl2, 10 µL 5 mg ml^{-1} DNase (D4527; Sigma), and 10 µL 10 mg ml^{-1} RNase (R5500; Sigma) were added, and mixtures were incubated for 20 min on ice. Cells were then disrupted by sonication (five pulses of 40 µm amplitude for 10 s), and membrane fraction pellets were collected by centrifugation (16,000 × g for 30 min at 4°C). Membranes were dissolved in 2 mL room temperature CoIP lysis buffer [50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (vol/vol) Triton X-100], chilled, and incubated for 30 min at 4°C; 80 µL anti-FLAG M2 affinity gel (A2220; Sigma) were added. The gel was washed before use five times with 0.5 mL 1× wash buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA) at room temperature as described in the manufacturer's protocol. The mixture of dissolved membranes and washed gel was added to a 2-mL gravity-flow column (Pierce) and incubated at 4°C overnight with gentle rotation. Lysate was removed, and the resin was washed three times with 1 mL CoIP lysis buffer at 25°C. FLAG-tagged protein was eluted from the column by incubation with 200 µL FLAG elution buffer (1× wash buffer containing 150 ng 3× FLAG peptide/µL) (F4799; Sigma) for 30 min at 4°C. Residual FLAG-tagged protein was eluted from the column by washing two times with 200 µL 1× wash buffer at 25°C. Eluates were filtered and concentrated (to ~40 µL) through 100-kDa cut-off Microcon columns (Millipore) by centrifugation (10,000 × g at RT). Concentrated samples were split evenly into two parts, and each one was mixed with 20 µL 2 × Laemmli sample buffer containing 5% (vol/vol) β-mercaptoethanol. One half was heated for 1 h at 95°C to remove crosslinks, while the other was kept intact. Samples were separated on 4-12% gradient SDS-PAGE gels in MES buffer and blotted into PVDF membranes, ready for immunoblotting with different antibodies (monoclonal anti-Flag and polyclonals anti-GFP, anti-Pbp2B, anti-MreB and anti-DivIVA). Finally, the membrane were developed using the Pierce ECL 2 Western Blotting substrate reagent. Chemiluminescence was detected using an ImageQuant LAS4000mini GE Healthcare system.

Supplementary Figures

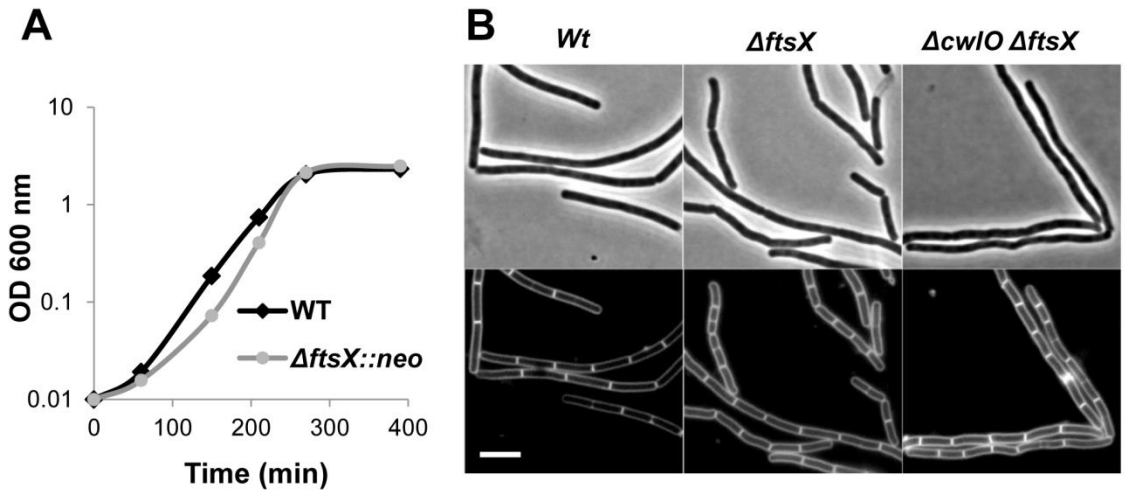

Figure S1. Phenotypes associated with *ftsX* and *cwlO* mutant strains. (A) Growth curve (logarithmic scale) of the wt and *fstX* null strain in liquid medium. **(B)** Cell morphologies of typical fields of strains Bs168CA, 4501 (*ΔftsX::neo*) and PDC465 (*ΔftsX::neo ΔcwlO::spec*). Scale bar represents 4 µm.

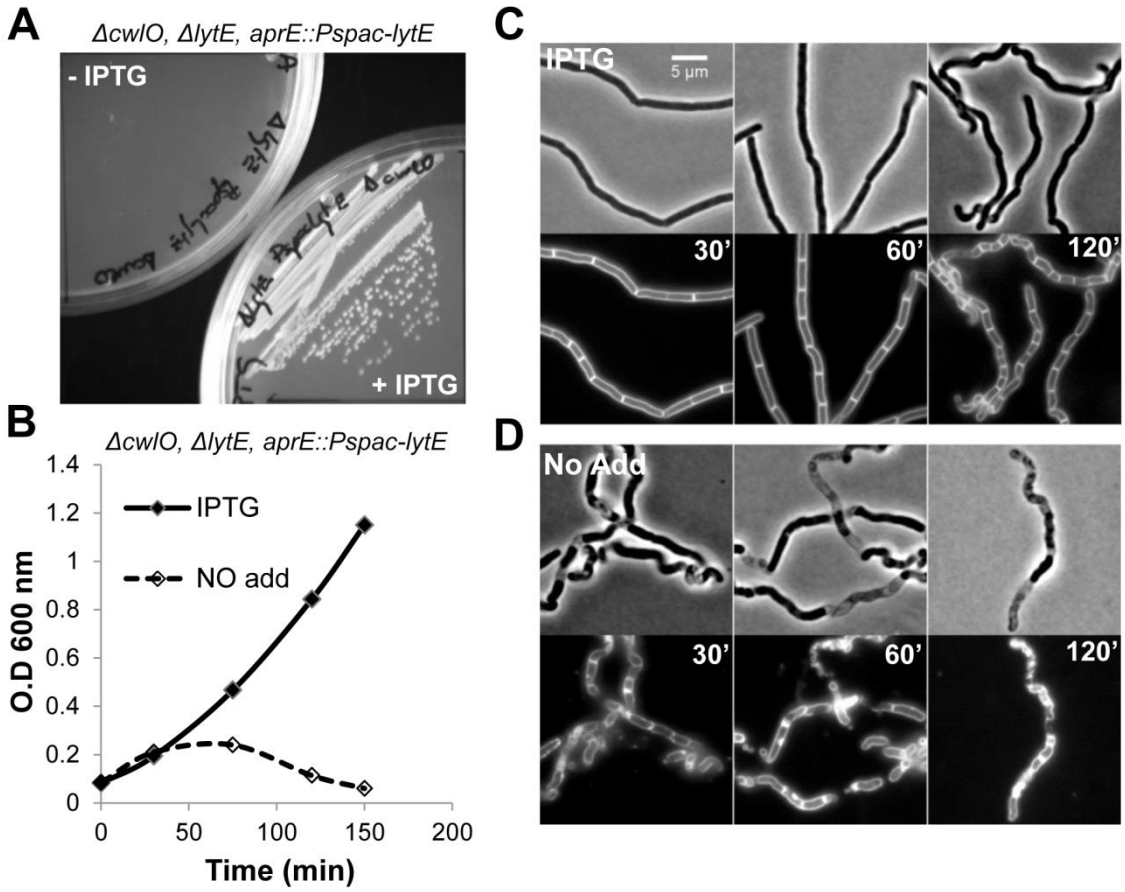

Figure S2. LytE and CwlO mutants are synthetically lethal. **(A)** Growth of strain PDC493 (*ΔcwlO::spec ΔlytE::cat aprE::P$_{spac}$-lytE)* on NA plates with or without 0.5 mM IPTG. **(B)** Growth of strain PDC493 on LB liquid medium in the presence or absence of IPTG. Growth curves (IPTG 0.5 mM, closed symbols; no addition, open symbols). **(C-D)** Effect of LytE depletion on cell morphology. Phase contrast micrographs and the corresponding membrane staining images were taken at the indicated times during the growth curves in (B). (C) 0.5 mM IPTG added, (D) No addition. The cell membranes were stained with FM5-95 membrane dye. Scale bar represents 5 µm.

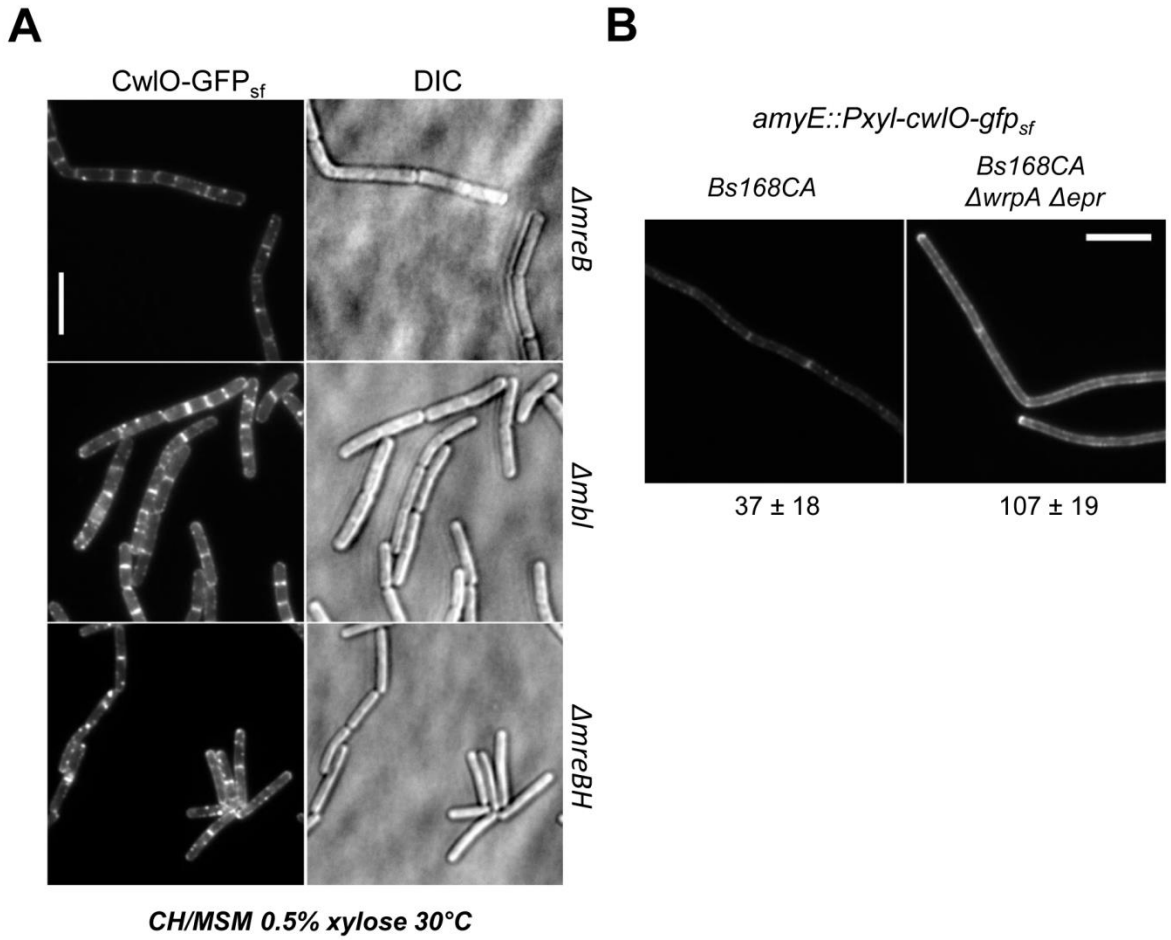

Figure S3. CwlO localization in different genetic backgrounds. DIC and epifluorescence microscopy of strains expressing the fluorescent fusion *amyE::*P_{xyl}*-cwlO-*gfp_{sf} . Cells were grown to mid-exponential phase in CH/MSM medium in the presence of 0.5% xylose at 30°C and immobilized on agarose-coated microscope slides. **(A)** The different panels correspond to strains PDC550 (*ΔmreB*), PDC552 (*Δmbl*) and PDC554 (*ΔmreBH*) as indicated. **(B)** CwlO-GFP_{sf} fluorescence is increased in a surface protease-deficient background. Cells of strains PDC519 and PDC528 (*ΔwrpA Δepr)* were grown in CH media at 30°C in the presence of 0.5% xylose. Fluorescent images were taken with the same acquisition settings and exposure times with the average relative fluorescence intensity over the lateral wall of the cells indicated below. An average 2- to 3-fold increase of the brightness of the CwlO-GFP fusion in the protease-deficient relative to the wild-type background was measured in four independent experiments.

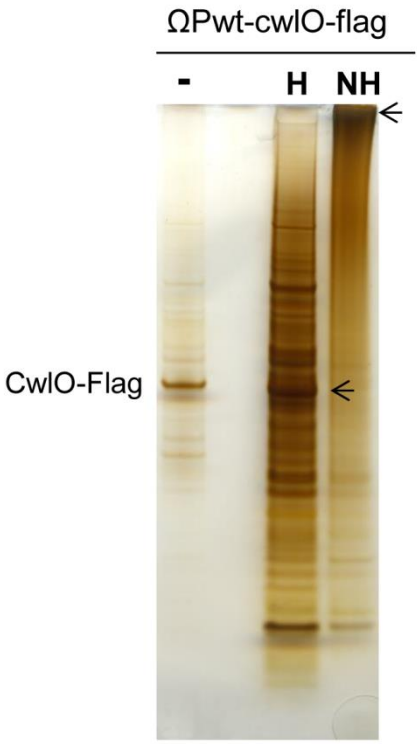

Figure S4. FtsX and CwlO interact within the same protein complex at the cell membrane. Pull down of cross-linked CwlO-Flag complexes in membranes detected by silver staining. Left lane, extract control sample before cross-linking. Right lanes, cross-linked samples that were heated (H) or not (NH) to break cross-links or maintain the complexes, respectively. The most prominent band in both lanes is labelled with an arrow, indicating the position of the CwlO-FLAG protein free or as part of a macro-molecular complex.

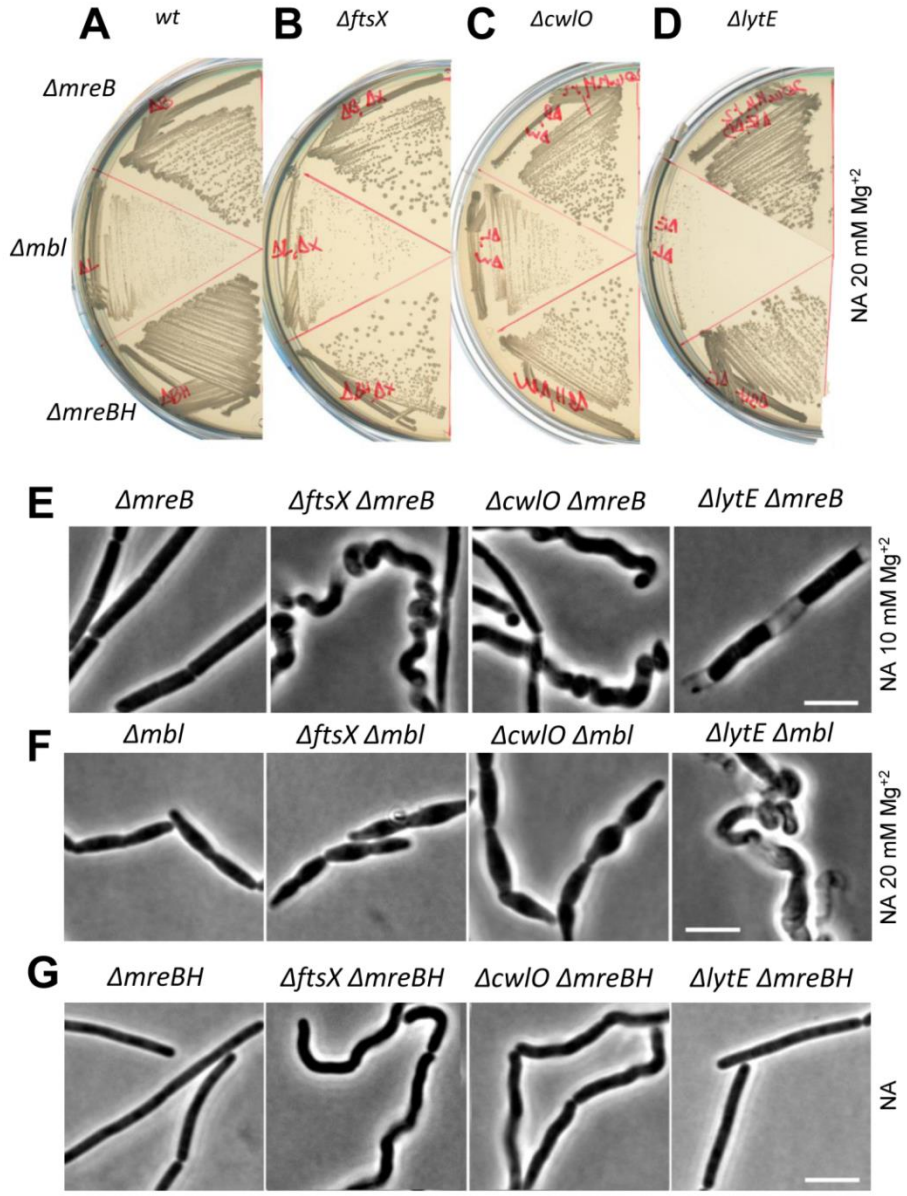

Figure S5. LytE is synthetically lethal with Mbl (A) Growth of strains *4281 (Ωcat 3427 ΔmreB), 4261 (Δmbl::cat) and* 4262 *(ΔmreBH::ery), and* the corresponding double mutant combinations with *ΔftsX:neo* **(B)***, ΔcwlO::spec* **(C)** *and Δ*l*ytE::tet* **(D)** gene deletions on NA plates in the presence of 20 mM Mg^{+2}. **(E)** Cell morphologies of typical fields of *mreB* mutant strain (4281) and derivatives in (B-D), growing in the presence of 10 mM of Mg^{+2}. Scale bar represents 5 μm. PDC454 (*ΔftsX:neo Ωcat 3427 ΔmreB*), PDC483 (*ΔcwlO::spec Ωcat 3427 ΔmreB*) and PDC577 (*ΔlytE::cat::tet ΔmreB::cat*. **(F)** Cell morphologies of typical fields of *mbl* mutant strain (4261) and derivatives in (B-D), growing in the presence of 20 mM of Mg^{+2}. Scale bar represents 5 μm. PDC453 (*ΔftsX:neo Δmbl*::cat), PDC473 (*ΔcwlO::spec Δmbl*::cat) and PDC576 (*ΔlytE::cat::tet Δmbl::cat*) **(G)** Cell morphologies of typical fields of *mreBH* mutant strain (4262) and derivatives in (B-D), growing on NA plates. Scale bar represents 5 μm. PDC467 *(ΔftsX:neo ΔmreBH:ery*), PDC471 (*ΔcwlO::spec ΔmreBH*:ery) *and* PDC470 *(Δ*l*ytE::tet ΔmreBH*:ery).

Supplementary tables

Table S1. E-values and identity homologies identified using the basic local alignment search tool (BLAST) (Altschul *et al.*, 1990).

B. subtilis	FtsE	FtsX	CwlO	LytE
E. coli	FtsE	FtsX	EnvC	YdhO
	1e-67	3e-22	2e-9	2.47e-19
	(46%)	(28%)	(20%)	(30%)
S. pneumoniae	FtsE	FtsX	PcsB	SPD_104
	1e-111	1e-64	1e-16	1.5e-05
	(66%)	(38%)	(30%)	(28%)
B. anthracis	FtsE	FtsX	BA5427	BA1952
	4.85e-76	2.19e-98	2.4e-39	9.29e-32
	(65%)	(60%)	(25%)	(29%)

Table S2. Bacterial strains used in this study.

***B. subtilis* strains**	**Relevant Genotype**	**Reference**
Bs168CA	*trpC2*	Barbe *et al.*, 2009
1A792	*trpC2 ΔlytABC::neo ΔlytD::tet ΔlytE::cam ΔlytF::spc*	Margot *et al.*, 1998
BP079	*trpC2 ΔcwlO::spc*	Bisicchia *et al.*, 2007
WE1	*trpC2 wprA::neo epr::tet*	Yamamoto *et al.*, 2003
WB800	*nprE aprE epr bpr mpr::ble nprB::bsr Δvpr wprA::hyg*	Wu *et al.*, 2002
2535	Bs168ED *ΔmreBH::spc*	Carballido-Lopez *et al.*, 2006
4261	Bs168CA *Δmbl::cat*	Schirner & Errington, 2009
4262	Bs168CA *ΔmreBH::erm*	Schirner & Errington, 2009
4281	Bs168CA *Ωcat 3427 ΔmreB*	Formstone & Errington, 2005
4501	Bs168CA *ΔftsX::neo*	This study
4502	Bs168CA *ΔftsEX::neo*	This study
4503	Bs168CA *ΔftsE::neo*	This study
PDC453	Bs168CA *ΔftsX::neo Δmbl::cat*	This study
PDC454	Bs168CA *Ωcat 3427 ΔmreB ΔftsX::neo*	This study
PDC463	Bs168CA *ΔcwlO::spc*	This study
PDC464	Bs168CA *ΔlytE::cat*	This study
PDC465	Bs168CA *ΔcwlO::spc ΔftsX::neo*	This study
PDC467	Bs168CA *ΔmreBH::erm ΔftsX::neo*	This study
PDC470	Bs168CA *ΔmreBH::erm ΔlytE::cat*	This study
PDC471	Bs168CA *ΔmreBH::erm ΔcwlO::spc*	This study
PDC472	Bs168CA *ΔlytE::cat aprE::P_{spac}-lytE erm*	This study
PDC473	Bs168CA *ΔcwlO::spc Δmbl::cat*	This study
PDC478	Bs168CA *ΔftsX::neo aprE::P_{spac}-lytE erm*	This study
PDC479	Bs168CA *ΔcwlO::spc aprE::P_{spac}-lytE erm*	This study
PDC480	Bs168CA *ΔftsX::neo::spc*	This study
PDC483	Bs168CA *ΔcwlO::spc Ωcat 3427 ΔmreB*	This study
PDC484	Bs168CA *ΔftsEX::neo::spc*	This study
PDC492	Bs168CA *ΔlytE::cat ΔftsX::neo aprE::P_{spac}-lytE erm*	This study
PDC493	Bs168CA *ΔlytE::cat ΔcwlO::spc aprE::P_{spac}-lytE erm*	This study

PDC519	Bs168CA *amyE::P_{xyl}-cwlO-gfp_{sf} spc*	This study
PDC528	PDC538 *amyE::P_{xyl}-cwlO-gfp_{sf} spc*	This study
PDC538	Bs168CA *wprA::hyg epr::tet*	This study
PDC540	Bs168CA *aprE::PrpsD-gfp spc*	This study
PDC541	Bs168CA *ΔftsX::neo aprE::PrpsD-gfp spc*	This study
PDC550	PDC528 *Ωcat 3427 ΔmreB*	This study
PDC552	PDC528 *Δmbl::cat*	This study
PDC554	PDC528 *ΔmreBH::erm*	This study
PDC560	PDC528 *ΔftsX::neo*	This study
PDC567	Bs168CA *amyE::P_{xyl}-cwlO spc*	This study
PDC575	Bs168CA *ΔlytE::cat::tet*	This study
PDC576	Bs168CA *ΔlytE::cat::tet Δmbl::cat*	This study
PDC577	Bs168CA *Ωcat 3427 ΔmreB ΔlytE::cat::tet*	This study
PDC590	PDC567 *ΔftsX::neo*	This study
PDC591	PDC567 *Ωcat 3427 ΔmreB*	This study
PDC592	PDC567 *Δmbl::cat*	This study
PDC593	PDC567 *ΔmreBH::erm*	This study
PDC594	PDC528 *ΔftsE::neo*	This study
PDC609	PDC538 *ΩPwt-cwlO-flag erm*	This study
PDC610	PDC538 *ΔftsX::neo ΩPwt-cwlO-flag erm*	This study
PDC612	*PDC538 ΩPwt-cwlO-flag erm amyE::P_{xyl}-ftsEX-gfp spc*	This study
PDC613	*PDC538 amyE::P_{xyl}-ftsEX-gfp spc*	This study
PDC620	Bs168CA *aprE::P_{spac}-lytE erm*	This study
PDC627	Bs168CA *ΔmreBH::spc*	This study
PDC632	Bs168CA *aprE::P_{xyl}-ftsEX erm*	This study
PDC639	Bs168CA *ΔcwlO::spc aprE::P_{xyl}-cwlO erm*	This study
PDC642	YK1119 *ΔlytE::cam::tet*	This study
PDC643	Bs168 *trpC2 Δmbl ΔmreBH ΔmreB::neo amyE::P_{spacHY}-mreBH cat* (BH-only)	This study
PDC650	YK1119 *ΔftsEX::neo::spc aprE::P_{xyl}-ftsEX erm*	This study
PDC651	YK1119 *ΔcwlO::spc aprE::P_{xyl}-cwlO erm*	This study

PDC659	PDC643 *ΔcwlO::spc aprE::P_{xyl}-cwlO erm*	This study
PDC660	Bs168 *trpC2 Δmbl ΔmreBH ΔmreB::neo amyE::P_{spacHY}-mreB cat* (B-only)	This study
PDC662	PDC660 *ΔcwlO::spc aprE::P_{xyl}-cwlO erm*	This study
PDC664	PDC660 *ΔftsEX::neo::spc aprE::P_{xyl}-ftsEX erm*	This study
PDC665	PDC538 *ΔftsE::neo ΩPwt-cwlO-flag erm*	This study
PDC678	YK1119 *ΔlytE::cam::tet, aprE::P_{xyl}--lytE erm*	This study
PDC687	Bs168CA *ΔlytE::cam::spc*	This study
PDC688	PDC660 *ΔlytE::cam::spc, aprE::P_{xyl}-lytE erm*	This study
PDC696	Bs168CA *ΔlytE::cam aprE::PrpsD-gfp spc*	This study
PDC697	PDC643 *ΔlytE::cam::spc, aprE::P_{xyl}-lytE erm*	This study
PDC702	Bs168CA *ΔlytE::cam::spc, aprE::P_{xyl}-lytE erm*	This study
PDC713	Bs168CA *Ωcat 3427 ΔmreB amyE::P_{xyl}-ftsE-gfp spc*	This study
PDC714	Bs168CA *Δmbl::cat amyE::P_{xyl}-ftsE-gfp spc*	This study
PDC715	Bs168CA *ΔmreBH::ery amyE::P_{xyl}-ftsE-gfp spc*	This study
YK1012	Bs168 *trpC2 Δmbl ΔmreBH*	Kawai *et al.*, 2009
YK1119	Bs168 *trpC2 Δmbl ΔmreBH ΔmreB::neo amyE::P_{spacHY}-mbl cat* (BL-only)	Kawai *et al.*, 2011
***E. coli* strains**	**Relevant characteristics**	**Reference**
BTH101	F- *glnV44 recA1 endA gyrA96 thi-1 hsdR17 spoT1 rfbD1 cya-854*	Karimova *et al.*, 1998
DH5α	F-*endA1 hsdR17 supE44 thi-1* λ-*recA1gyrA96 relA1* D(*lacZYA-argF*)*U169* φ*80 dlacZ* D*M15*	GIBCO-BRL
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lac*Iq*Z_M15* Tn*10* (Tet)]	Stratagene Ltd.

spc, spectinomycin; kan, kanamycin; erm, erythromycin; neo, neomycin; cat, chloramphenicol; tet, tetracyclin; ble, bleomycin; bsr, blasticidin S; hyg, hygromycin. Other abbreviations: *Δ*, deletion; Ω, insertion. *gfpmut1*: F64L, S65T variant of GFP (GFPmut1; (Cormack *et al.*, 1996)). *gfp_{sf}*: variant of GFP (Pedelacq *et al.*, 2006**)**

Table S3. Plasmids used in this study.

Plasmid	Relevant Genotype	Reference or source
pMUTin4	*bla erm lacZ lacI*	Vagner *et al.*, 1998
pMUTin-flag	*bla erm* P_{spac}-FLAG *lacI*	Kaltwasser *et al.*, 2002
pAPNC213	bla aprE::P_{spac}-mcs spc	Morimoto *et al.*, 2002
pBEST501	*bla neo*	Itaya *et al.*, 1989
pSG1728	bla amyE::P_{xyl}-mcs	Lewis & Marston, 1999
pSG1154	*bla amyE*:: *P_{xyl}-gfpmut1 spc*	Lewis & Marston, 1999
pUT18::zip	*P_{lac}-zip-cyaA*$^{675\text{-}1197}$ *bla*	Karimova *et al.*, 1998
pKT25::zip	*P_{lac}-cyaA*$^{675\text{-}1197}$*-zip bla*	Karimova *et al.*, 1998
pUT18C	*P_{lac}-cyaA*$^{675\text{-}1197}$-mcs *bla*	Karimova *et al.*, 1998
pKT25	*P_{lac}-cyaA*$^{1\text{-}732}$-mcs *kan*	Karimova *et al.*, 1998
pAPNC213-erm	bla aprE::P_{spac}-MCS erm	Olmedo-Verd, E. Unpublished
pUC57-gfp-sf	bla gfp-sf	Murray, H. Unpublished
pAPNC-PrpsD-gfp spc	pAPNC-P_{rpsD}-gfp spc	This study
pAPNC-PrpsD-gfp erm	pAPNC-P_{rpsD}-gfp erm	This study
pAPNC-erm-LytE	pAPNC-P_{spac}-Pwt-LytE erm	This study
pSG1728-cwlO	pSG1728-P_{xyl}-cwlO spc	This study
pSG1728-ftsEX	pSG1728-P_{xyl}-ftsEX spc	This study
pSG-Pxyl-cwlO-gfp_{sf}	pSG1728-P_{xyl}-cwlO-gfp_{sf} spc	This study
pSG1154-Pxyl-ftsEX-gfp	pSG1154-P_{xyl}-ftsEX-gfp_{sf} spc	This study
pMUTin-‘cwlO-flag	pMUTin-P_{spac}‘cwlO-flag erm	This study
pAPNC-P_{xyl}-cwlO	pAPNC213-P_{xyl}-cwlO erm	This study
pAPNC-P_{xyl}-ftsEX	pAPNC213-P_{xyl}-ftsEX erm	This study
pAPNC-P_{xyl}-lytE	pAPNC213-P_{xyl}-lytE erm	This study
pKT25::*ftsEX*	*P_{lac}-cyaA*$^{1\text{-}732}$-ftsEX *kan*	This study
pKT25::ftsE	*P_{lac}-cyaA*$^{1\text{-}732}$-ftsE *kan*	This study
pKT25::*ftsX*	*P_{lac}-cyaA*$^{1\text{-}732}$-ftsX *kan*	This study
pUT18::*ftsE*	*P_{lac}*-ftsE-*cyaA*$^{675\text{-}1197}$ *bla*	This study
pUT18::*ftsX*	*P_{lac}*-ftsX-*cyaA*$^{675\text{-}1197}$ *bla*	This study
pUT18::*ftsEX*	*P_{lac}*-ftsEX-*cyaA*$^{675\text{-}1197}$ *bla*	This study

MCS: Multi-cloning site, spc, spectinomycin; kan, kanamycin; erm, erythromycin; neo, neomycin; bla, ampicillin;

Table S4. Primers used in this study

Name	Sequence (5'-3')
54-GFPBamHIF	CGGGATCCGCAAACTAATGTGCAACTTAC
54-gfp-BglIIrev	GCAGATCTTTTGTATAGTTCATCCATGCC
amyE-F1	ACCACCAGTGATTATGCC
AmyEGFPsftoAprERevBamHI	CTGGATCCGCGCCGACCTTGAC
amyE-R1	TGCATAACTGCTTCCAAC
AmyEtoAprERevBamHI	CTGGATCCCGCTCTAGAACTAGTGGATCTG
AprEForw	CTCTACGGAAATAGCGAGAG
AprERev	AGAAGCAGGTATGGAGGAAC
CwlOCterFHindIIIFlag	CGTAAGCTTGCTCATCTGATGATTCTTC
CwlO-FXhoI	CATCTCGAGCAAATGAGGACAGGTTCACAG
cwlORev-EcoRI	GGAATTCTTGAACAACACGTCTTACAACAC
CwlOrevKpnIFlag	GGGGTACCTTGTTGAACAACACGTCTTAC
CwlORevSacI	GATCGAGCTCTACTTGAACAACACGTCTTACAAC
ForA	TGAATCCCACGAAGAATTACAAATGACTCATG
ForB1	ATAAAGTGAAAAAGGATCCCGTTTTCGGGACG
ForB2	CAAGAGGGGAGTATGGATCCTATGATTAAAAT
ForEXba1	AAGATTTCTAGATTTCATGATAGAGATGAAGGAAG
ForXXba1	CAAGATCTAGATATGGTTCATATGATTAAAATTCT
FtsEFxhoI	CCGCTCGAGGATATAAAAGATTAGGTGATTTC
FtsErevEcoRI	GGAATTCATCATATGAACCATACTCCC
FtsEupF5	GCTGAAGGCCAAGCTGTATC
FtsXdown3R	AATCTCTTTAAGCACAAAGAAACAG
FtsXdown5FXbaI	CATCTAGAGAAAAAGCCGTTCCGTTTTCG
FtsXRevEcoRI	GGAATTCTACTCGCAGAAACTTGCGG
GFP-sf-FEcoRI	GGAATTCACAAACATGTCAAAAGGAG
GFPsf-RevSpeI-NotI	CATCATAGCGGCCGCGCCGACCTTGACTAGTGCTC
LytEFEcoRI	GGAATTCATCGAATCTTTTCGCACCGAG
lytEFNheI	GATGCTAGCGTTAACATTTGGGGAGG
LytEF-xhoI	CGTCTCGAGGTTAACATTTGGGGAGG
LytEF-xmaI	TATCCCGGGGAGGAAAATATGAAAAAG
LytE-NcoIF	CTGACCATGGGAGTTAACATTTGGGGAG
LytERev-KpnI	CTGGTACCGAATCTTTTCGCACCGAGG
LytERevSacI	CTGAGCTCGACATCGAATCTTTTCGCACCG
LytERevSphI	GCACGCATGCTAGAATCTTTTCGCACCGAG
p+lytExhoI	CCGCTCGAGGTTATCTTGCCTTATTTGATG
pAPNC213-F1	TCACTCTCAAGGCTACACAGG
pAPNC213-F2	CTACAAGGTGTGGCATAATGTG
pAPNC213-R1	GGTATGGAGGAACCTGCTTC
Pxyl-FSphI	GTGACATTTGCATGCTTCAAAG
RevA1	TCACCTAATCGCATGCATCATTTTATCTATCA
RevA2	TCACGCAAGTGGCATGCGAGAATTTTAATCAT
RevB	ACAGACACTATCTCTCACCGCCTCAAGCCAAA
RevEKpnI	GCGCCCGAGGTACCTAATCATATGAACCATAC
RevXKpnI	CGGCTTTTGGTACCTATACTCGCAGAAACTTGCGG

Supplementary References

Altschul, S. F., W. Gish, W. Miller, E. W. Myers & D. J. Lipman, (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403-410.

Anagnostopoulos, C. & J. Spizizen, (1961) Requirements for Transformation in *Bacillus subtilis*. *J Bacteriol* **81**: 741-746.

Barbe, V., S. Cruveiller, F. Kunst, P. Lenoble, G. Meurice, A. Sekowska, D. Vallenet, T. Wang, I. Moszer, C. Medigue & A. Danchin, (2009) From a consortium sequence to a unified sequence: the *Bacillus subtilis* 168 reference genome a decade later. *Microbiology* **155**: 1758-1775.

Bisicchia, P., D. Noone, E. Lioliou, A. Howell, S. Quigley, T. Jensen, H. Jarmer & K. M. Devine, (2007) The essential YycFG two-component system controls cell wall metabolism in *Bacillus subtilis*. *Mol Microbiol* **65**: 180-200.

Carballido-Lopez, R., A. Formstone, Y. Li, S. D. Ehrlich, P. Noirot & J. Errington, (2006) Actin homolog MreBH governs cell morphogenesis by localization of the cell wall hydrolase LytE. *Dev Cell* **11**: 399-409.

Cormack, B. P., R. H. Valdivia & S. Falkow, (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**: 33-38.

Cutting, S. M., and P. B. Vander Horn. , (1990) Genetic analysis. In: Molecular biological methods for Bacillus. C. R. H. a. S. M. Cutting (ed). England: John Wiley & Sons Ltd. Chichester, , pp. p. 27-74.

Dinh, T. & T. G. Bernhardt, (2011) Using superfolder green fluorescent protein for periplasmic protein localization studies. *J Bacteriol* **193**: 4984-4987.

Formstone, A. & J. Errington, (2005) A magnesium-dependent mreB null mutant: implications for the role of *mreB* in *Bacillus subtilis*. *Mol Microbiol* **55**: 1646-1657.

Hamoen, L. W., W. K. Smits, A. de Jong, S. Holsappel & O. P. Kuipers, (2002) Improving the predictive value of the competence transcription factor (ComK) binding site in *Bacillus subtilis* using a genomic approach. *Nucleic Acids Res* **30**: 5517-5528.

Itaya, M., K. Kondo & T. Tanaka, (1989) A neomycin resistance gene cassette selectable in a single copy state in the *Bacillus subtilis* chromosome. *Nucleic Acids Res* **17**: 4410.

Kaltwasser, M., T. Wiegert & W. Schumann, (2002) Construction and application of epitope- and green fluorescent protein-tagging integration vectors for *Bacillus subtilis*. *Appl Environ Microbiol* **68**: 2624-2628.

Karimova, G., J. Pidoux, A. Ullmann & D. Ladant, (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci U S A* **95**: 5752-5756.

Kawai, Y., K. Asai & J. Errington, (2009) Partial functional redundancy of MreB isoforms, MreB, Mbl and MreBH, in cell morphogenesis of *Bacillus subtilis*. *Mol Microbiol* **73**: 719-731.

Kawai, Y., J. Marles-Wright, R. M. Cleverley, R. Emmins, S. Ishikawa, M. Kuwano, N. Heinz, N. K. Bui, C. N. Hoyland, N. Ogasawara, R. J. Lewis, W. Vollmer, R. A. Daniel & J. Errington, (2011) A widespread family of bacterial cell wall assembly proteins. *EMBO J* **30**: 4931-4941.

Lewis, P. J. & A. L. Marston, (1999) GFP vectors for controlled expression and dual labelling of protein fusions in *Bacillus subtilis*. *Gene* **227**: 101-110.

Margot, P., M. Wahlen, A. Gholamhoseinian, P. Piggot & D. Karamata, (1998) The *lytE* gene of *Bacillus subtilis* 168 encodes a cell wall hydrolase. *J Bacteriol* **180**: 749-752.

Morimoto, T., P. C. Loh, T. Hirai, K. Asai, K. Kobayashi, S. Moriya & N. Ogasawara, (2002) Six GTP-binding proteins of the Era/Obg family are essential for cell growth in *Bacillus subtilis*. *Microbiology* **148**: 3539-3552.

Pedelacq, J. D., S. Cabantous, T. Tran, T. C. Terwilliger & G. S. Waldo, (2006) Engineering and characterization of a superfolder green fluorescent protein. *Nat Biotechnol* **24**: 79-88.

Sambrook, J., E. F. Fritsch & T. Maniatis, (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory.

Schirner, K. & J. Errington, (2009) The cell wall regulator {sigma}I specifically suppresses the lethal phenotype of *mbl* mutants in *Bacillus subtilis*. *J Bacteriol* **191**: 1404-1413.

Vagner, V., E. Dervyn & S. D. Ehrlich, (1998) A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology* **144 (Pt 11)**: 3097-3104.

Wu, S. C., J. C. Yeung, Y. Duan, R. Ye, S. J. Szarka, H. R. Habibi & S. L. Wong, (2002) Functional production and characterization of a fibrin-specific single-chain antibody fragment from *Bacillus subtilis*: effects of molecular chaperones and a wall-bound protease on antibody fragment production. *Appl Environ Microbiol* **68**: 3261-3269.

Yamamoto, H., S. Kurosawa & J. Sekiguchi, (2003) Localization of the vegetative cell wall hydrolases LytC, LytE, and LytF on the *Bacillus subtilis* cell surface and stability of these enzymes to cell wall-bound or extracellular proteases. *J Bacteriol* **185**: 6666-6677.